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Award Number: DAMD17-99-1-9441

TITLE: Extracellular Matrix Regulations of Membrane Type 1-Matrix Metalloproteinasis (MT1-MMP) and Matrix Metalloproteinase-2 (MMP-2) in Human Breast Fibroblasts

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REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (1 Jul 99 - 1 Jul 00)

4. TITLE AND SUBTITLE
Extracellular Matrix Regulations of Membrane Type 1-Matrix Metalloproteinasis (MT1-MMP) and Matrix Metalloproteinase-2 (MMP-2) in Human Breast Fibroblasts

5. FUNDING NUMBERS
DAMD17-99-1-9441

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Previous studies have shown that MT1-MMP (MMP-14) initiates pro-MMP-2 activation in a process that is tightly regulated by the level of TIMP-2. However, given the difficulty in modulating TIMP-2 levels, the direct effect of TIMP-2 on MT1-MMP processing and on pro-MMP-2 activation in a cellular system could not be established. Here, recombinant vaccinia viruses encoding full-length MT1-MMP or TIMP-2 were used to express MT1-MMP alone or in combination with various levels of TIMP-2 in mammalian cells. We show that TIMP-2 regulates the amount of active MT1-MMP (57 kDa) on the cell surface whereas in the absence of TIMP-2 MT1-MMP undergoes autocatalysis to a 44-kDa form, which displays a N-terminus starting at G^{285} and hence lacks the entire catalytic domain. Neither pro-MT1-MMP (N terminus S^{24}) nor the 44-kDa form bound TIMP-2. In contrast, active MT1-MMP (N-terminus Y^{112}) formed a complex with TIMP-2 suggesting that regulation of MT1-MMP processing is mediated by a complex of TIMP-2 with the active enzyme. Consistently, TIMP-2 enhanced the activation of pro-MMP-2 by MT1-MMP. Thus, under controlled conditions, TIMP-2 may act as a positive regulator of MT1-MMP activity by promoting the availability of active MT1-MMP on the cell surface and consequently, may support pericellular proteolysis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Pericellular Proteolysis, Matrix Metalloproteinases	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

For my thesis research, I have been investigating the regulation of two members of the Matrix Metalloproteinase (MMPs) family, MMP-2 and MT1-MMP and the role of their natural inhibitors, namely, Tissue Inhibitor of Matrix Metalloproteinases (TIMPs). Since these enzymes are produced by fibroblasts, I tried to study their regulation in fibroblasts isolated from breast tissue. However, the manipulation of the fibroblasts turned to be very elusive. This difficulty let us to choose a vaccinia expression system to study the regulation of these matrix metalloproteinases in an eukaryotic system, which provides us with a more controllable system. This step corresponds to **Task 1** on the proposal that states :
"Parallel studies to determine the processing of MT1-MMP and the role of TIMP-2 in MMP-2 activation will be performed in a vaccinia expression system in which mammalian cells are infected and transfected with full length human MT1-MMP cDNA."

My project has dealt with a key aspect of breast cancer metastasis and had involved the use of a variety of techniques including cell biology and biochemistry in a relevant experimental model.

Body:

Turnover of extracellular matrix (ECM) is a fundamental process of many physiological and pathological conditions. A major group of enzymes responsible for ECM degradation is the matrix metalloproteinases (MMPs). The MMPs are multidomain zinc-dependent endopeptidases that, with few exceptions, share a basic structural organization comprising a propeptide, catalytic, hinge, and C-terminal (hemopexin-like) domains (1,2). All MMPs are produced in a latent form (pro-MMP) requiring activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of tissue inhibitors of metalloproteinases (TIMPs) that bind to the active site inhibiting catalysis (3).

Over the last five years, the MMP family has been expanded to include a new subfamily of membrane-tethered MMPs known as membrane-type MMPs (MT-MMPs), which as of today includes six enzymes: MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMP (4-9). The MT-MMPs are unique because they are anchored to the plasma membrane (PM). MT1-MMP (MMP-14) was the first member of the MT-MMP family to be discovered and was identified as the first physiological activator of pro-MMP-2 (gelatinase A) (4,10). The role of MT1-MMP in pericellular proteolysis is not restricted to pro-MMP-2 activation as MT1-MMP is a functional enzyme that can also degrade a number of ECM components (11) and hence can play a direct role in ECM turnover. MT1-MMP has also been shown to be a key enzyme in tumor metastasis and angiogenesis (12).

Perhaps the most interesting aspect of MT1-MMP is the nature of its interactions with TIMP-2 and the role they play in pro-MMP-2 activation. Studies using PM extracts containing MT1-MMP (10) or using a transmembrane-deleted form of MT1-MMP (13) have shown that, at low concentrations, TIMP-2 stimulates pro-MMP-2 activation whereas at high concentrations it inhibits activation. Based on these observations, a model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-terminal portion of TIMP-2, leaving the negatively charged C-terminal region of TIMP-2 available for the binding of the hemopexin-like domain of pro-MMP-2 (10). This complex, referred to as the "ternary complex", has been suggested to cluster pro-MMP-2 at the cell surface near a TIMP-2-free active MT1-MMP molecule, which is thought to initiate activation of the bound pro-MMP-2. Pro-MMP-2 activation would occur only at low TIMP-2 concentrations relative to MT1-MMP, which would permit availability of active MT1-MMP to activate the pro-MMP-2 bound in the ternary complex. Thus, under restricted conditions, TIMP-2 is thought to promote the activation process by acting as a molecular link between MT1-MMP and pro-MMP-2. However, this model of pro-MMP-2 activation had not been established in a living cell system due to the difficulty in modulating the level of TIMP-2 expression.

Conflicting results have been reported regarding the MT1-MMP form(s) responsible for the formation of the MT1-MMP/TIMP-2 complex (10, 14-17). In fact, only a few studies examined the direct binding of TIMP-2 to full-length MT1-MMP. Strongin *et al.* (10) reported the binding of MT1-MMP from phorbol ester-treated HT-1080 PM to TIMP-2 bound to an MMP-2-C-terminal domain-affinity column. The bound MT1-MMP turned out to be the active enzyme starting at Y^{112}. Using radiolabeled TIMP-2 and PM extracts derived from COS-1 cells transfected with the full-length MT1-MMP cDNA, Zucker *et al.* (15) showed the formation of a ~80-kDa cross-linking product. However, the nature of the cross-linked MT1-MMP form(s) was not determined. Other studies showed the cross-linking of radiolabeled TIMP-2 with a purified autoactivated recombinant MT1-MMP lacking the TM domain (18). However, binding of TIMP-2 to pro-MT1-MMP has not been examined. Finally, a 56-kDa form of MT1-MMP lacking the prodomain, found in the culture media of breast cancer cells, co-purified with TIMP-2 (19). Although various sources of MT1-MMP were used, these results were consistent with the active form of MT1-MMP being responsible for the binding of TIMP-2. Nonetheless, conflicting results were recently reported by Cao *et al.* (17), who proposed that the prodomain of MT1-MMP is essential for the binding of TIMP-2 to MT1-MMP and that pro-MT1-MMP can activate pro-MMP-2.

A major limitation in the study of MT1-MMP-TIMP-2 interactions has been the source of MT1-MMP, which included truncated enzymes and enzymes expressed in bacteria (13,20). Furthermore, PM extracts (10,14) and mammalian cells transfected with the MT1-MMP cDNA (4,17) contain endogenous TIMPs and/or MMPs. Thus, the direct contribution of TIMP-2 to pro-MMP-2 activation and its effect on MT1-MMP processing could not be clearly assessed. Therefore, we chose a vaccinia virus expression system in mammalian cells (21-23), which allowed control of the level of TIMP-2 expression in the cells, to investigate the effect of various TIMP-2 levels on MT1-MMP processing and pro-MMP-2 activation and to identify the nature of the major MT1-MMP species detected.

Purpose: The goal of this first phase was to study the regulation of MT1-MMP and MMP-2 and the role of a particular inhibitor , namely TIMP-2 in this process. Since these matrix metalloproteinases are overexpressed in many cancers, including breast cancer, it is relevant to study the role of TIMPs in the processing of MT1-MMP and MMP-2 activation. I hypothesize d that TIMP-2 will affect the processing of MT1-MMP and its activation of pro-MMP-2. The purpose of these studies is to examine in detail the regulatory mechanisms underlying the processing of MT1-MMP and its activation of pro-MMP-2 to understand how these processes contribute to the degradation of ECM during cancer metastasis.

Objective: To investigate the effect of TIMP-2 on the processing of MT1-MMP and pro-MMP-2 activation .

Key Research Accomplishments:

- A cellular approach designed to express MT1-MMP in the absence or presence of TIMP-2 facilitated the characterization of the four major MT1-MMP species and revealed a unique interaction between MT1-MMP and TIMP-2.
- We showed that TIMP-2 regulates the amount of active MT1-MMP (57 kDa) on the cell surface.
- In the absence of TIMP-2, MT1-MMP undergoes autocatalysis to a 44-kDa form, which displays a N-terminus starting at G^{285} and hence lacks the entire catalytic domain.
- Neither pro-MT1-MMP (N terminus S^{24}) nor the 44-kDa form bound TIMP-2. In contrast, active MT1-MMP (N-terminus Y^{112}) formed a complex with TIMP-2 suggesting that regulation of MT1-MMP processing is mediated by a complex of TIMP-2 with the active enzyme.
- TIMP-2 enhanced the activation of pro-MMP-2 by MT1-MMP.

- Our data suggest that the 57-kDa species of MT1-MMP is the major pro-MMP-2 activator. This species is the active enzyme form, as determined by N-terminal sequencing, and its appearance correlated with enhanced pro-MMP-2 activation.

List of Reportable Outcomes:

- The results of this research were published in the Journal of Biological Chemistry (JBC) Vol 275, No.16, Issue of April 21, pp.12080-12089, 2000 (**Reprint Attached**).

- Presentation of a poster in the Molecular Aspects of Metastasis Meeting held in Snowmass, Colorado in September, 1999.
  Role Of Timp-2 In MT1-MMP Processing And Pro-MMP-2 Activation
  Sonia Hernandez-Barrantes, Marta Toth, Margarida Bernardo, David Gervasi and Rafael Fridman
  Department of Pathology, Wayne State University and Karmanos Cancer Institute, Detroit, MI 48201.

Conclusions:

Our results demonstrated that the concentration of active MT1-MMP (57 kDa) on the cell surface was directly and positively regulated by TIMP-2.
Absence of inhibitor, on the other hand, resulted in a significant decrease in the amount of active enzyme on the cell surface leading to the generation of a membrane-bound inactive 44-kDa species, which was further processed to lower molecular weight forms.
Based on the results in the vaccinia system, which allowed modulation of the level of TIMP-2 expression in the cells, we can conclude that the accumulation of the 57-kDa species of MT1-MMP and the reduction in the amount of the 44-kDa forms is a sole consequence of the presence of TIMP-2. The data obtained with this study show for the first time in a living cellular system that pro-MMP-2 activation by wild-type MT1-MMP is tightly regulated by the level of TIMP-2 expression in the cells.
Binding of TIMP-2 to activated MT1-MMP (57 kDa) inhibited autocatalysis and consequently, active enzyme accumulated on the cell surface as cells produced more MT1-MMP. This suggested that, under conditions of low levels of TIMP-2 expression relative to MT1-MMP, TIMP-2 may act as a positive regulator of MT1-MMP activity and therefore may enhance pericellular proteolysis including pro-MMP-2 activation and ECM degradation. Excess TIMP-2 will eventually block all active MT1-MMP inhibiting proteolysis. This model reflects the dynamic and unique interactions between MT1-MMP and TIMP-2 in living cells that tightly regulate MT1-MMP pericellular activity.

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Appendix:

See Reprint Attached. *J.Biol.Chem.***275**, 12080-12089

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 16, Issue of April 21, pp. 12080–12089, 2000
Printed in U.S.A.

Binding of Active (57 kDa) Membrane Type 1-Matrix Metalloproteinase (MT1-MMP) to Tissue Inhibitor of Metalloproteinase (TIMP)-2 Regulates MT1-MMP Processing and Pro-MMP-2 Activation*

(Received for publication, July 13, 1999, and in revised form, January 21, 2000)

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Previous studies have shown that membrane type 1-matrix metalloproteinase (MT1-MMP) (MMP-14) initiates pro-MMP-2 activation in a process that is tightly regulated by the level of tissue inhibitor of metalloproteinase (TIMP)-2. However, given the difficulty in modulating TIMP-2 levels, the direct effect of TIMP-2 on MT1-MMP processing and on pro-MMP-2 activation in a cellular system could not be established. Here, recombinant vaccinia viruses encoding full-length MT1-MMP or TIMP-2 were used to express MT1-MMP alone or in combination with various levels of TIMP-2 in mammalian cells. We show that TIMP-2 regulates the amount of active MT1-MMP (57 kDa) on the cell surface whereas in the absence of TIMP-2 MT1-MMP undergoes autocatalysis to a 44-kDa form, which displays a N terminus starting at Gly^{285} and hence lacks the entire catalytic domain. Neither pro-MT1-MMP (N terminus Ser^{24}) nor the 44-kDa form bound TIMP-2. In contrast, active MT1-MMP (N terminus Tyr^{112}) formed a complex with TIMP-2 suggesting that regulation of MT1-MMP processing is mediated by a complex of TIMP-2 with the active enzyme. Consistently, TIMP-2 enhanced the activation of pro-MMP-2 by MT1-MMP. Thus, under controlled conditions, TIMP-2 may act as a positive regulator of MT1-MMP activity by promoting the availability of active MT1-MMP on the cell surface and consequently, may support pericellular proteolysis.

Turnover of extracellular matrix (ECM)[1] is a fundamental process of many normal and pathological conditions. A major group of enzymes responsible for ECM degradation is the matrix metalloproteinases (MMPs). The MMPs are multidomain zinc-dependent endopeptidases that, with few exceptions, share a basic structural organization comprising a propeptide, catalytic, hinge, and C-terminal (hemopexin-like) domains (1, 2). All MMPs are produced in a latent form (pro-MMP) requiring activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of tissue inhibitors of metalloproteinases (TIMPs) (3).

Over the last five years, the MMP family has been expanded to include a new subfamily of membrane-tethered MMPs known as membrane-type MMPs (MT-MMPs), which as of today includes five enzymes: MT1-, MT2-, MT3-, MT4-, and MT5-MMP (4–8). The MT-MMPs, with the exception of MT4-MMP (9), are unique because they are anchored to the plasma membrane (PM) by means of a hydrophobic stretch of approximately 20 amino acids leaving the catalytic domain exposed to the extracellular space. MT1-MMP (MMP-14) was the first member of the MT-MMP family to be discovered and was identified as the first physiological activator of pro-MMP-2 (gelatinase A) (4, 10). The role of MT1-MMP in pericellular proteolysis is not restricted to pro-MMP-2 activation as MT1-MMP is a functional enzyme that can also degrade a number of ECM components (11–14) and hence can play a direct role in ECM turnover. MT1-MMP has been recently shown to be the first member of the MMP family indispensable for normal growth and development since mice deficient in MT1-MMP exhibit a variety of connective tissue pathologies and a short life span (15). MT1-MMP has also been shown to be a key enzyme in tumor metastasis and angiogenesis (16, 17).

Perhaps the most interesting aspect of MT1-MMP is the nature of its interactions with TIMP-2 and the role they play in pro-MMP-2 activation. Studies using PM extracts containing MT1-MMP (10) or a transmembrane-deleted form of MT1-MMP (18) have shown that, at low concentrations, TIMP-2 stimulates pro-MMP-2 activation whereas at high concentrations it inhibits activation. In addition, cross-linking experiments using PM preparations demonstrated the binding of TIMP-2 to MT1-MMP and to the hemopexin-like domain of pro-MMP-2 (10). Based on these observations, a model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-terminal portion of TIMP-2, leaving the negatively charged C-terminal region of TIMP-2 available for the binding of the hemopexin-like domain of pro-MMP-2 (10, 19). This ternary complex has been suggested to cluster pro-MMP-2 at the cell surface near a TIMP-2-free active MT1-MMP molecule, which is thought to initiate activation of the bound pro-MMP-2. Pro-MMP-2 activation would occur only at low TIMP-2 concentrations relative to

* This work was supported by National Institutes of Health Grant CA-61986 and Department of Defense Grant DAMD17-99-1-9440 (to R. F.), National Insititues of Health Grant CA-78646 (to Q-X. S.), and Department of Defense Predoctoral Fellowship DAMD17-99-1-9441 (to S. H-B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody; pAb, polyclonal antibody; pfu, plaque-forming units; FBS, fetal bovine serum; ECL, enhanced chemiluminescence; PM, plasma membrane; MT, membrane-type.

MT1-MMP, which would permit availability of active MT1-MMP to activate the pro-MMP-2 bound in the ternary complex. Thus, under restricted conditions, TIMP-2 is thought to promote the activation process by acting as a molecular link between MT1-MMP and pro-MMP-2. Kinetic studies with PM containing MT1-MMP (19) and studies with the catalytic domain of MT1-MMP immobilized on agarose beads (18) have supported this model. However, this model of pro-MMP-2 activation has not been established in a living cell system due to the difficulty in modulating the level of TIMP-2 expression.

Conflicting results have been reported regarding the MT1-MMP form(s) responsible for the formation of the MT1-MMP·TIMP-2 complex (10, 19–22). In fact, only a few studies examined the direct binding of TIMP-2 to full-length MT1-MMP. Strongin *et al.* (10) reported the binding of MT1-MMP from phorbol ester-treated HT-1080 PM to TIMP-2 bound to an MMP-2-C-terminal domain-affinity column. The bound MT1-MMP turned out to be the active enzyme starting at Tyr^{112}. Using radiolabeled TIMP-2 and PM extracts derived from COS-1 cells transfected with the full-length MT1-MMP cDNA, Zucker *et al.* (20) showed the formation of a ~80-kDa cross-linking product. However, the nature of the cross-linked MT1-MMP form(s) was not determined. Other studies showed the cross-linking of radiolabeled TIMP-2 with a purified autoactivated recombinant MT1-MMP lacking the transmembrane domain (13). However, binding of TIMP-2 to pro-MT1-MMP has not been examined. Finally, a 56-kDa form of MT1-MMP lacking the prodomain, found in the culture media of breast cancer cells, co-purified with TIMP-2 (23). Although various sources of MT1-MMP were used, these results were consistent with the active form of MT1-MMP being responsible for the binding of TIMP-2. Nonetheless, conflicting results were recently reported by Cao *et al.* (22), who proposed that the prodomain of MT1-MMP is essential for the binding of TIMP-2 to MT1-MMP and that pro-MT1-MMP can activate pro-MMP-2.

A major limitation in the study of MT1-MMP-TIMP-2 interactions has been the source of MT1-MMP, which included truncated enzymes and enzymes expressed in bacteria (18, 24). Furthermore, PM extracts (10, 19) and mammalian cells transfected with the MT1-MMP cDNA (4, 22, 25) contain endogenous TIMPs and/or MMPs. Thus, the direct contribution of TIMP-2 to pro-MMP-2 activation and its effect on MT1-MMP processing could not be clearly assessed. Here, a vaccinia virus expression system in mammalian cells (26–28), which allowed control of the level of TIMP-2 expression in the cells, was used to investigate the effect of various TIMP-2 levels on MT1-MMP processing and pro-MMP-2 activation and to identify the nature of the major MT1-MMP species detected.

EXPERIMENTAL PROCEDURES

Buffers—The following buffers were used: collagenase buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl, and 0.02% Brij-35), lysis buffer (25 mM Tris-HCl, pH 7.5, 1% IGEPAL CA-630: a non-ionic detergent from Sigma, 100 mM NaCl, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride), HNTG buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% IGEPAL, and 10% glycerol), harvest buffer (60 mM Tris-HCl, pH 7.5, 0.55% SDS, and 2 mM EDTA), and buffer R (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Brij-35, and 1% (v/v) dimethyl sulfoxide).

Recombinant Vaccinia Viruses and Cell Culture—The production of the recombinant vaccinia virus (vTF7-3) expressing bacteriophage T7 RNA polymerase has been described by Fuerst *et al.* (28). Recombinant vaccinia viruses, expressing either human TIMP-2 (vSC59-T2) or TIMP-1 (vT7-T1) were obtained by homologous recombination, as described previously (28, 29). To construct the recombinant vaccinia virus expressing human MT1-MMP, the full-length MT1-MMP cDNA (a generous gift from Dr. G. Goldberg, Washington University, St. Louis, MO) was amplified by polymerase chain reaction and then cloned into the pTF7EMCV-1 expression vector, under control of the T7 promoter (30). After sequence verification of the insert in both directions, the resulting pTF7EMCV-1-MT1 plasmid was used to generate a recombinant vaccinia virus containing the full-length human MT1-MMP cDNA (vT7-MT1) by homologous recombination with wild type vaccinia virus, as described previously (26, 28). Non-malignant monkey kidney epithelial BS-C-1 (CCL-26) and human fibrosarcoma HT-1080 (CCL-121) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. HeLa S3 cells were obtained from ATCC (CCL-2.2) and grown in suspension in minimal essential Spinner medium (Quality Biologicals, Inc., Gaithersburg, MD) supplemented with 5% horse serum. All other tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY).

Recombinant Proteins and Antibodies—Human pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as described previously (26, 27, 31). The anti-TIMP-2 mAb CA101 (32) and a rabbit pAb to MT1-MMP (here referred to as pAb 437) (33) have been previously described. The rabbit pAb 160 and pAb 36 to MT1-MMP were generated and characterized, as described previously (34).

Infection-Transfection and Pulse-Chase Analysis of MT1-MMP—BS-C-1 cells in 6-well plates were infected with 30 plaque forming units (pfu)/cell of vTF7-3 for 45 min in Dulbecco's modified Eagle's medium containing 2.5% FBS. The media were then removed and the infected cells were transfected with 2 μg/ml of the pTF7EMCV-1-MT1 plasmid in Opti-MEM (Life Technologies, Inc.) (1 ml/well) using LipofectAMINE (Life Technologies, Inc.), as described by the manufacturer. After an incubation of 3.5 h, the medium was aspirated and replaced with 1 ml/well of Dulbecco's modified Eagle's medium without methionine supplemented with L-glutamine, 25 mM HEPES, pH 7.5, 0.1% dialyzed FBS, and 500 μCi/ml [^{35}S]methionine (NEN Life Science Products Inc., Wilmington, DE). After a 15-min pulse, the plates were placed on ice, the medium was aspirated and the cells received 1 ml/well of chase media (Opti-MEM with 0.1% dialyzed FBS, 25 mM HEPES, pH 7.5, and 4.8 mM methionine). At the end of the chase period (0–120 min at 37 °C), the medium was aspirated and the cells were lysed with 100 μl/well of harvest buffer. The lysates were then subjected to five cycles of boiling and freezing followed by a brief centrifugation. The supernatants were collected into clean tubes with the addition of 5 mM iodoacetamide, 2.5% Triton X-100, and 20 μg/ml aprotinin (final concentrations). For immunoprecipitations, lysates were incubated (16 h, 4 °C) with 5 μg of the pAb 437 to MT1-MMP followed by addition of 30 μl of protein G-Sepharose beads for an additional overnight incubation at 4 °C. After recovering the beads by brief centrifugation, the supernatants were discarded and the beads were washed five times with cold HTNG buffer. The immunoprecipitates were recovered from the beads with 4× Laemmli sample buffer (35), boiled, and resolved by SDS-PAGE under reducing conditions followed by autoradiography.

Expression of MT1-MMP and TIMP-2 by Infection—To express MT1-MMP, BS-C-1 cells (10^6 cells/35-mm well) in 6-well plates were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 for 45 min in 0.5 ml/well of Dulbecco's modified Eagle's medium containing 2.5% FBS (infection medium) at 37 °C. In some experiments, the pfu/cell of the vT7-MT1 virus was varied (0–10 pfu/cell) to modulate the level of MT1-MMP expression. To co-express MT1-MMP with TIMP-2, and to modulate the level of inhibitor expression, BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 and increasing amounts (0–10 pfu/cell) of vSC59-T2 for 45 min in 0.5 ml/well of infection medium at 37 °C. To co-express MT1-MMP and TIMP-1, the vSC59-T2 virus was replaced with vT7-T1. As a control, BS-C-1 cells were infected with the vTF7-3 virus alone at 5 pfu/cell. After the infection, the virus containing-medium was aspirated and each well received 2-ml of fresh infection medium without viruses. The infected cells were then incubated for a minimum of 6 h at 37 °C, before any further experimentation.

Activation of Pro-MMP-2—At the indicated times following infection, the medium was aspirated and replaced with 1 ml/well of Opti-MEM media (Life Technologies, Inc.) containing 2 nM recombinant pro-MMP-2. The cells were then incubated for various times at 37 °C. The medium was collected and clarified by a brief centrifugation (2,000 × *g*, 5 min) and the cells were solubilized in 100 μl/well of cold lysis buffer and centrifuged (13,000 × *g*) for 15 min at 4 °C. Samples of the medium (5 μl) and lysates (5 μl) were mixed with 4× Laemmli sample buffer (35) without reducing agents and without heating and subjected to gelatin zymography, as described previously (36).

Immunoblot Analysis—Infected BS-C-1 cells in 6-well plates, as described above, were lysed in cold lysis buffer (100 μl/well). The lysates (20 μl from each sample) were mixed with 4× Laemmli sample buffer

with β-mercaptoethanol and then resolved by reducing SDS-PAGE followed by immunoblot analysis using the appropriate antibodies. The immune complexes were detected using the ECL system (Pierce, Rockford, IL), according to the manufacturer's instructions.

Surface Biotinylation—Twenty-four hours post-infection, BS-C-1 cells in 150-mm tissue culture dishes were surface-biotinylated with 0.5 mg/ml of the water-soluble, cell impermeable, biotin analog sulfo-NHS-biotin (Pierce) for 30 min at 4 °C in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM). The biotinylation reaction was quenched (10 min at 4 °C) with a freshly prepared solution of 50 mM NH_4Cl in PBS-CM. After two washes with cold PBS-CM, the biotinylated cells were solubilized in harvest buffer (2 ml/150-mm dish), as described above. The lysates were then boiled (5 min), centrifuged (13,000 × *g*, 5 min) and the supernatants were supplemented with 2.5% Triton X-100 (final concentration). The lysates (500 μl) were immunoprecipitated with the appropriate antibody and protein G-Sepharose beads. The immunoprecipitates were resolved by reducing SDS-PAGE followed by blotting to a nitrocellulose membrane. The biotinylated proteins were detected with streptavidin-horseradish peroxidase and ECL.

Isolation of PM—BS-C-1 cells were infected with vaccinia viruses to express MT1-MMP alone or MT1-MMP with TIMP-2, as described above. The next day, the medium was aspirated and the cells were scraped into cold 25 mM Tris-HCl, 50 mM NaCl, pH 7.4. The samples were centrifuged (800 × *g*, 5 min) twice at 4 °C. The pellets were resuspended in the same buffer containing 8.5% sucrose, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 4 mM benzamidine, and 10 mM *N*-ethylmaleimide. After sonication, the solutions were centrifuged at 19,000 × *g* at 4 °C for 30 min. The supernatants were collected and centrifuged at 100,000 × *g* at 4 °C for 1.25 h. The pellets were resuspended in the same buffer containing protease inhibitors and no sucrose. The PM fractions of HT-1080 cells treated (12 h, 37 °C) with 100 nM phorbol ester were prepared as described previously (36, 37).

Activation of Pro-MMP-2 by PM of BS-C-1 Cells Co-expressing MT1-MMP and TIMP-2—Pro-MMP-2 (55 nM) was incubated at 37 °C with each PM fraction (0.15 μg/μl) in a total volume of 200 μl of collagenase buffer. At varying times, 20 μl of the reaction mixture were added to acrylic cuvettes containing 2 ml of a 7 μM solution of the fluorescence quenched substrate MOCAcPLGLA_2pr(Dnp)-AR-NH_2 (Peptides International, Louisville, KY) (38) in buffer R, at 25 °C (31). Substrate hydrolysis was monitored with a Photon Technology International spectrofluorometer interfaced to a Pentium computer, equipped with the RatioMaster™ and FeliX™ hardware and software, respectively. The cuvette compartment was maintained at 25 °C. Fluorescence measurements were carried out at excitation and emission wavelengths of 328 and 393 nm and excitation and emission band passes of 1 and 3 nm, respectively. Less than 10% hydrolysis of the fluorogenic peptide substrate was monitored, as described by Knight (39). At the PM concentrations used, hydrolysis of this substrate by MT1-MMP was insignificant when compared with the hydrolysis by MMP-2.

Coupling of TIMP-2 to Affi-Gel 10 Matrix—Purified recombinant TIMP-2 (200 μg) in PBS was allowed to bind to 200 μl of Affi-Gel 10 (Bio-Rad) for 1 h at 22 °C with rotation. To block any active esters, 20 μl of 1 M ethanolamine-HCl, pH 8, were added to the reaction mixture followed by a 1-h incubation at 22 °C. The matrix (Affi-Gel 10-TIMP-2) was allowed to settle and the supernatant was subjected to reducing 12% SDS-PAGE to determine the amount of uncoupled TIMP-2. The Affi-Gel 10-TIMP-2 matrix was washed four times with PBS and equilibrated in collagenase buffer. The immobilized TIMP-2 maintained its capability to bind pro-MMP-2, as determined by SDS-PAGE analysis of the bound enzyme.

Binding of MT1-MMP to Immobilized TIMP-2—BS-C-1 cells in 150-mm dishes were infected to express MT1-MMP alone as described above. The cells were solubilized in lysis buffer (2 ml/dish) and the lysates (500 μl) were incubated (12 h, 4 °C) with a 50-μl suspension of either Affi-Gel 10-TIMP-2 matrix or Affi-Gel 10 matrix. The samples were centrifuged (13,000 × *g*, 5 min) and the supernatant (unbound fraction) and the matrix (bound fraction) were collected. The matrix beads were washed (5 times) with HNTG buffer. The beads and the unbound fraction (20 μl) were mixed with 20 μl of 4× Laemmli sample buffer with β-mercaptoethanol. The samples were then resolved by 12% SDS-PAGE followed by immunoblot analysis with the pAb 437, as described above.

Co-immunoprecipitation of MT1-MMP with TIMP-2—A pulse-chase experiment of MT1-MMP biosynthesis was carried out as described above except that the BS-C-1 cells were lysed in lysis buffer after the chase. Aliquots (20 μl) of the ^{35}S-lysates were then incubated with or without unlabeled TIMP-2 (150 nM) for 2 h at 4 °C followed by immunoprecipitation with anti-TIMP-2 mAb CA101 and protein G-Sepharose beads, as described above. In parallel, another aliquot (20 μl) of the lysates received 10× harvest buffer followed by immunoprecipitation with the pAb 437 to anti-MT1-MMP, as described above. The immunoprecipitates were resolved by reducing SDS-PAGE followed by autoradiography.

Immunoaffinity Purification and N-terminal Sequencing of MT1-MMP Species—ImmunoPure Immobilized Protein A beads (Pierce) (1 ml) were incubated (1 h, 22 °C) with 2 ml of the pAb 437 serum in binding buffer (0.01 M sodium phosphate, pH 7.5–8.0, containing 150 mM NaCl). The beads were washed with 0.2 M sodium borate, pH 9.0, followed by coupling of the antibodies with 20 mM dimethyl pimelimidate hydrochloride in the same buffer for 30 min at 22 °C. The beads were then centrifuged (5 min, 2000 × *g*) and the supernatant was aspirated. The beads were resuspended in a solution of 0.2 M ethanolamine, pH 8.0, followed by a 2-h incubation with gentle rocking at room temperature. The pAb 437/Protein A beads were washed with binding buffer. BS-C-1 cells in eight 150-mm dishes infected to co-express MT1-MMP and TIMP-2 were lysed in harvest buffer. The lysate was supplemented with 2.5% Triton X-100 (final concentration) and incubated (12 h, 4 °C) with the pAb 437/Protein A beads. After a brief centrifugation, the beads were collected and washed three times with harvest buffer supplemented with 2.5% Triton X-100. The bound proteins were eluted with sample buffer, boiled, and subjected to reducing 10% SDS-PAGE in 1.5-mm wide gel cassettes and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The transferred proteins were stained with 0.1% Coomassie Blue Brilliant R-250 in 1% acetic acid, 40% methanol. An aliquot of the eluted proteins was also subjected to immunoblot analysis using the pAb 437. After identification, the corresponding bands were cut out from the polyvinylidene difluoride membrane and sent for microsequencing to ProSeq (Boxford, MA).

RESULTS

Biosynthesis of Recombinant MT1-MMP and Activation of Pro-MMP-2—To examine the expression and biosynthesis of MT1-MMP, BS-C-1 cells were infected with the vTF7-3 vaccinia virus and then transiently transfected with the pTF7EMCV-1-MT1 plasmid. After the infection-transfection procedure, the processing of MT1-MMP was examined by pulse-chase analysis followed by immunoprecipitation with the pAb 437. As shown in Fig. 1*A*, MT1-MMP was synthesized as a ~60-kDa protein that was sequentially processed to a major 57-kDa form and to a heterogeneous species of 44–40 kDa. Cleavage to the 57-kDa form was clearly detected after a 30-min chase period. After 45 min, the 44–40-kDa forms were detected. After 90 min, a ~35-kDa product was also immunoprecipitated by the pAb 437. In vTF7-3-infected but non-transfected BS-C-1 cells (Fig. 1*A*, *NT*), endogenous MT1-MMP was not detected demonstrating the lack of expression of endogenous enzyme in BS-C-1-infected cells.

The pTF7EMCV-1-MT1 plasmid was used to construct a recombinant vaccinia virus expressing full-length human MT1-MMP (vT7-MT1) by homologous recombination, as described previously (26, 28). BS-C-1 cells were co-infected with vTF7-3 and increasing pfu/cell of vT7-MT1 and then examined for their ability to activate exogenous pro-MMP-2. Generation of active MMP-2 in the media and the lysates was monitored by gelatin zymography. As shown in Fig. 1*B*, co-infection of BS-C-1 cells with vTF7-3 and vT7-MT1 resulted in pro-MMP-2 processing to the ~64-kDa intermediate form and the 62-kDa active species (14) in both the media and cell lysates. In contrast, BS-C-1 cells infected with the vTF7-3 virus alone failed to initiate pro-MMP-2 activation despite a small amount of autocatalytically generated MMP-2 present in the pro-MMP-2 stock solution. A very weak ~57-kDa gelatinolytic band was also detected in the lysates of cells co-infected with the two viruses but not with vTF7-3 alone. The 57-kDa form is derived from MT1-MMP, as will be discussed below. In the absence of exogenous pro-MMP-2, infected BS-C-1 cells showed no detectable levels of endogenous pro-MMP-2 (data not shown), as previously reported (26, 27).

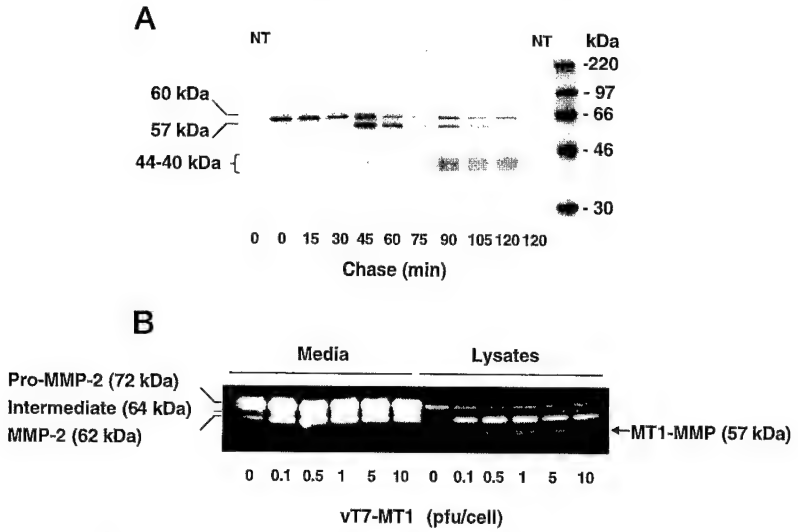

FIG. 1. **Processing and activity of MT1-MMP.** *A*, BS-C-1 cells were infected (45 min) with 30-pfu/cell of vTF7-3 and then transfected with 2 μg/well of the pTF7EMCV-1-MT1 expression vector. At 3.5 h post-infection transfection, the cells were pulse-labeled with [^{35}S]methionine for 15 min and then chased for 0–120 min. At the indicated times, the cells were lysed in harvest buffer and the lysates were immunoprecipitated with the pAb 437 and protein G-Sepharose beads. The immunoprecipitates were resolved by 10% SDS-PAGE under reducing conditions followed by autoradiography. *NT*, BS-C-1 cells infected with the vTF7-3 virus but not transfected and processed at 0 and 120 min. *B*, BS-C-1 cells were infected with 5 pfu/cell of vTF7-3 or co-infected with 5 pfu/cell of vTF7-3 and increasing pfu/cell (0.1–10) of vT7-MT1 to express MT1-MMP. At 12 h post-infection, the cells were incubated with 2 nM exogenous pro-MMP-2 for an additional 7 h at 37 °C. The medium (5 μl) and lysates (5 μl) were then harvested and analyzed by gelatin zymography.

Effects of TIMP-2 on Pro-MMP-2 Activation and MT1-MMP Processing—Previous *in vitro* studies demonstrated a role for TIMP-2 in pro-MMP-2 activation by MT1-MMP (10, 18, 19). However, the role of TIMP-2 in pro-MMP-2 activation has not been established in a cell-based assay. To investigate the role of TIMP-2 in MT1-MMP-dependent activation of pro-MMP-2, we used the vaccinia system to co-express MT1-MMP with TIMP-2 in BS-C-1 cells using the appropriate recombinant vaccinia viruses. To vary the level of inhibitor in the system, BS-C-1 cells were co-infected with various amounts (pfu/cell) of vSC59-T2, the TIMP-2-expressing vaccinia virus, while keeping constant the amount of vT7-MT1, thus varying the MT1-MMP/TIMP-2 ratio. It should be noted that all cells were infected with the polymerase-expressing virus (vTF7-3). After infection, the cells were incubated with exogenous pro-MMP-2. The lysates were then analyzed for MT1-MMP and TIMP-2 expression by immunoblot analysis (Fig. 2*A*) and pro-MMP-2 activation was monitored by gelatin zymography of the media and the lysates (Fig. 2*B*). As shown in the immunoblot of Fig. 2*A*, the level of TIMP-2 expression was dependent on the amounts (pfu/cell) of vSC59-T2 virus used to infect the cells. Under the same conditions, BS-C-1 cells infected to express MT1-MMP alone showed no detectable expression of endogenous TIMP-2 by immunoblot analysis (Fig. 2*A*) and by ELISA determination (data not shown), in agreement with our previous studies in vaccinia-infected cells (26, 27, 31).

Immunoblot analysis of the same lysates with the anti-MT1-MMP pAb 437 showed a profile of MT1-MMP forms that varied with the level of TIMP-2 expression. In the absence of TIMP-2, the lysates exhibited the 60- and 44–40-kDa species of MT1-MMP with the latter being the major species. Increased expression of TIMP-2 correlated with the accumulation of the 57-kDa species of MT1-MMP, which was concomitant with a gradual decrease in the 44–40-kDa forms. The 57-kDa species of MT1-MMP detected in the co-infected cells co-migrated with the MT1-MMP form present in the PM of phorbol ester-treated HT-1080 cells (Fig. 2*A*, *lane c*). Lack of a direct correlation between the intensity of the bands corresponding to the 44–40-kDa species and the intensity of the bands corresponding to the 60- and 57-kDa forms may be due to a differential solubilization of these forms from the cells when using Nonidet P-40 (IGEPAL) as detergent. Indeed, extraction with SDS (harvest buffer) significantly improved the yield and detection of the 60-kDa species of MT1-MMP (data not shown).

Zymographic analysis (Fig. 2*B*) showed that the activation of pro-MMP-2 by MT1-MMP, as determined by the appearance of the 62-kDa species of MMP-2, was dependent on the level of TIMP-2 expression. Low levels of TIMP-2 expression correlated with an enhanced activation of pro-MMP-2 when compared with cells expressing MT1-MMP alone in both the media and the cell lysates. On the other hand, high levels of TIMP-2 expression were associated with lower amounts of active MMP-2 (62 kDa). In the lysates of cells co-expressing MT1-MMP and TIMP-2, we also detected a weak ~57-kDa-gelatinolytic band (Fig. 2*B*), which was identified as MT1-MMP (see Fig. 3). Time course analysis (Fig. 2*C*) of the activation process by MT1-MMP co-expressed with TIMP-2, revealed the presence of active MMP-2 (62 kDa) in the lysates as early as 30 min after addition of pro-MMP-2. At the same time, the enzyme in the medium remained mostly in the latent form. With time, the 62-kDa species was also detected in the medium suggesting that after surface activation the 62-kDa form dissociates from the cell surface and it is released into the medium (Fig. 2*C*).

TIMP-2 but Not TIMP-1 Induces the Accumulation of the 57-kDa Form of MT1-MMP—As shown above (Fig. 2), co-expression of MT1-MMP and TIMP-2 induced the accumulation of the 57-kDa species of MT1-MMP, which exhibited gelatinolytic activity. To further investigate the effects of TIMP-2 on the accumulation of the 57-kDa species and to compare its effect with that of TIMP-1, BS-C-1 cells were infected to express MT1-MMP alone or MT1-MMP with increasing amounts of either TIMP-2 or TIMP-1. These experiments were carried out in the absence of exogenous pro-MMP-2. Generation of the 57-kDa species was monitored by gelatin zymography and immunoblot analysis. As shown in Fig. 3*A*, the intensity of the 57-kDa gelatinolytic species increased as a function of the amount (pfu/cell) of the vSC59-T2 virus (Fig. 3*A*, *lanes 4–6*). On the other hand, lysates of cells expressing MT1-MMP alone (Fig. 3*A*, *lane 3*) showed little or no detectable gelatinolytic bands. Co-expression of MT1-MMP with TIMP-1 had no effect

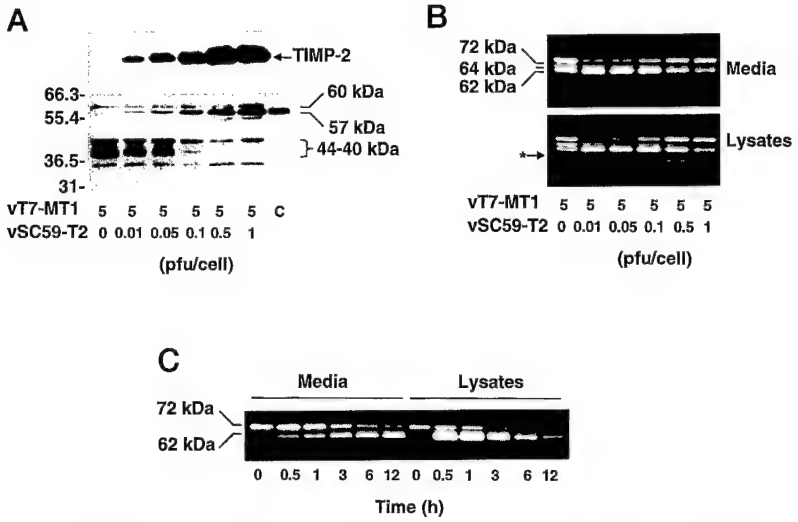

FIG. 2. **TIMP-2 enhances the activation of pro-MMP-2 by MT1-MMP and induces the accumulation of the 57-kDa species of MT1-MMP.** *A* and *B*, BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 and increasing amounts of vSC59-T2 (0–1 pfu/cell) to co-express MT1-MMP and TIMP-2. At 6 h post-infection, the cells were incubated with 2 nM exogenous pro-MMP-2 for an additional 12 h at 37 °C followed by solubilization of the cell monolayer with lysis buffer. In *A*, lysates (20 μl) were subjected to reducing 12% SDS-PAGE followed by immunoblot analysis using the anti-TIMP-2 mAb (CA101) (*upper panel*) and the anti-MT1-MMP pAb 437 (*lower panel*). Detection of the antigens was performed using ECL (Pierce). As control (*lane C*), PM fractions (16 μg) of phorbol ester-treated HT-1080 cells were similarly analyzed. In *B*, the media and lysates (5 μl each) were subjected to gelatin zymography. *Asterisk* shows the 57-kDa MT1-MMP enzyme. *C*, BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 and 0.01 pfu/cell of vSC59-T2. At 6 h post-infection, the cells were incubated with 2 nM exogenous pro-MMP-2 for various times (0–12 h). At each time, the medium and lysates were harvested and analyzed by gelatin zymography. These experiments were repeated at least three times with similar results.

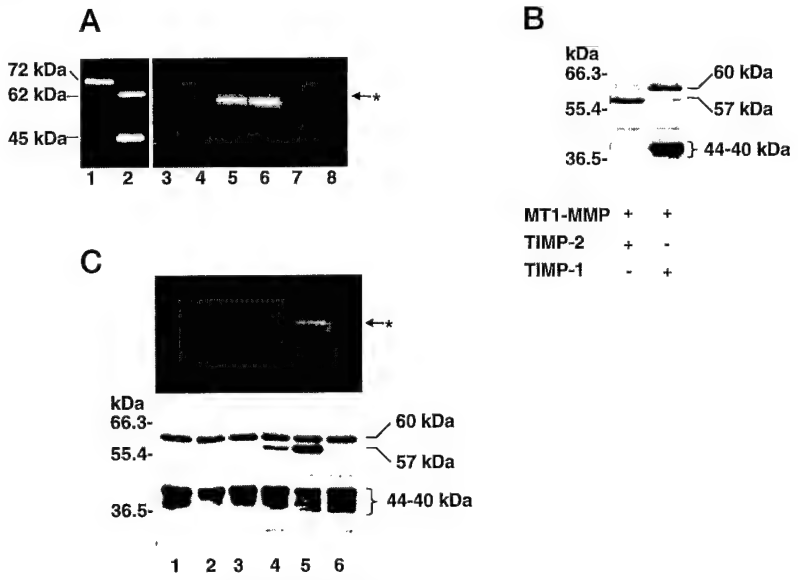

FIG. 3. **Effect of TIMP-2 and TIMP-1 on the profile of MT1-MMP forms.** *A*, BS-C-1 were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 (*lanes 3–8*) and increasing amounts of vSC59-T2 (*lane 4*, 0.01 pfu/cell; *lane 5*, 0.1 pfu/cell; *lane 6*, 1 pfu/cell) or vT7-T1 (*lane 7*, 0.01 pfu/cell; *lane 8*, 1 pfu/cell). At 12 h post-infection, the cells were harvested in 100 μl of lysis buffer and the lysates (20 μl) were analyzed by gelatin zymography. *Lane 1* shows purified pro-MMP-2 (5 ng) and *lane 2* shows active MMP-2 (62 and 45 kDa) (5 ng), as references. *B*, BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 and either vSC59-T2 (5 pfu/cell) to co-express TIMP-2 or vT7-T1 (5 pfu/cell) to co-express TIMP-1. The lysates were resolved by reducing 12% SDS-PAGE followed by immunoblot analysis using the pAb 437 and detection by ECL. *C*, BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1. After infection, the cells were incubated (12 h, 37 °C) without (*lane 1*) or with increasing amounts of either exogenous TIMP-2 (*lane 2*, 1 ng/ml; *lane 3*, 10 ng/ml; *lane 4*, 100 ng/ml; *lane 5*, 500 ng/ml) or TIMP-1 (*lane 6*, 500 ng/ml). The lysates were harvested and analyzed by gelatin zymography and immunoblot analysis with the pAb 437 to MT1-MMP. *Asterisks* in *A* and *C* show the 57-kDa MT1-MMP gelatinolytic enzyme.

on the gelatinolytic activity (Fig. 3*A*, *lanes 7* and *8*) and on the profile of the MT1-MMP forms (Fig. 3*B*) when compared with cells co-expressing MT1-MMP and TIMP-2 (Fig. 3*B*) or MT1-MMP alone (shown in Fig. 3*C*, *lane 1*).

The accumulation of the 57-kDa species was also observed after addition of exogenous TIMP-2 to cells expressing MT1-MMP. As shown in Fig. 3*C*, 500 ng of TIMP-2 (Fig. 3*C*, *lane 5*) clearly induced the appearance of the 57-kDa gelatinolytic form when compared with cells that did not receive the inhibitor (Fig. 3*C*, *lane 1*). Consistently, immunoblot analysis of the same lysates showed a dose-dependent accumulation of the 57-kDa species after addition of exogenous TIMP-2 (Fig. 3*C*, *lanes 4* and *5*). Under these conditions, reduction in the relative amounts of the 44–40-kDa species of MT1-MMP was not evident. This suggests that exogenous TIMP-2, as opposed to inhibitor co-expressed with MT1-MMP (Fig. 3*B*), is less effi-

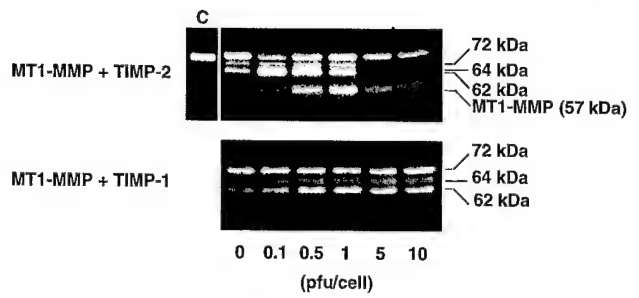

FIG. 4. **TIMP-2 but not TIMP-1 regulates the MT1-MMP-dependent activation of pro-MMP-2.** BS-C-1 cells were co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 and increasing amounts (0–10 pfu/cell) of either vSC59-T2 (MT1-MMP + TIMP-2) or vT7-T1 (MT1-MMP + TIMP-1). At 6 h post-infection, the cells were incubated with 2 nM exogenous pro-MMP-2 for an additional 10 h at 37 °C. The lysates were then harvested and analyzed by gelatin zymography. *Lane c* shows pro-MMP-2 incubated with BS-C-1 cells infected with 5 pfu/cell of vTF7-3 alone. These experiments were repeated at least three times with similar results.

cient in preventing the generation of the 44–40-kDa forms. Addition of exogenous TIMP-1 (500 ng) had no effect on the appearance of the 57-kDa gelatinolytic species and did not alter the profile of MT1-MMP forms, as determined by zymography and immunoblot analysis, respectively (Fig. 3*C, lane 6*).

We then compared the effects of TIMP-2 and TIMP-1 on the ability of MT1-MMP to initiate pro-MMP-2 activation. To this end, BS-C-1 cells were infected to express MT1-MMP with either TIMP-2 or TIMP-1 using the appropriate vaccinia viruses as described under "Experimental Procedures." After infection, the cells were examined for their ability to initiate pro-MMP-2 activation by gelatin zymography. As shown in Fig. 4, TIMP-2 enhanced the MT1-MMP-dependent activation of pro-MMP-2 at low inhibitor concentrations when compared with MT1-MMP alone. At high inhibitor concentrations (5–10 pfu/cell of vSC59-T2), a significant inhibition of pro-MMP-2 activation was observed. In addition, the presence of TIMP-2 correlated with the appearance of the 57-kDa species of MT1-MMP. In contrast to TIMP-2, co-expression of MT1-MMP with TIMP-1 had little or no effect on the rate of pro-MMP-2 activation (Fig. 4) and the 57-kDa form was not detected in the lysates.

Effect of TIMP-2 on the Cellular Distribution of MT1-MMP Forms—We investigated the effect of TIMP-2 on the nature of the MT1-MMP forms expressed on PM isolated from co-infected BS-C-1 cells. As shown in Fig. 5*A*, the PM of BS-C-1 cells expressing only MT1-MMP contained the 60- and 44–40-kDa forms, as determined by immunoblot analysis using the pAb 437 (Fig. 5*A, lane 3*). Co-expression of MT1-MMP and TIMP-2 correlated with the detection of both the 60- and 57-kDa forms in the PM whereas the amount of the 44–40-kDa species was significantly reduced (Fig. 5*A, lane 4*). PM of control cells (infected with vTF7-3 alone) showed neither MT1-MMP forms (Fig. 5*A, lane 2*), as expected. PM of phorbol ester-treated HT-1080 cells were analyzed in parallel and found to contain the 57-kDa form of MT1-MMP, as a major enzyme species (Fig. 5*A, lane 1*). The PM fractions were also examined for the presence of TIMP-2 (Fig. 5*B*). As expected, TIMP-2 was only detected in the PM of BS-C-1 cells co-expressing MT1-MMP and TIMP-2 (Fig. 5*B, lane 4*) and in the PM of HT-1080 cells (Fig. 5*B, lane 1*).

The surface association of MT1-MMP and TIMP-2 in BS-C-1 cells expressing MT1-MMP with or without TIMP-2 was examined by surface biotinylation followed by immunoprecipitation with the appropriate antibodies. The 60- and 44–40-kDa forms of MT1-MMP were the major surface-biotinylated species immunoprecipitated from cells expressing MT1-MMP alone (Fig. 5*C, lane 1*), in agreement with the results obtained with the PM fractions. As expected, no biotinylated endogenous TIMP-2 was immunoprecipitated from the same sample (Fig. 5*C, lane 3*). In the cells co-infected to express MT1-MMP and TIMP-2, the 57-kDa form of MT1-MMP was also surface-biotinylated, in addition to the 60- and 44–40-kDa forms (Fig. 5*C, lane 4*). TIMP-2 was also detected on the cell surface (Fig. 5*C, lane 6*) suggesting its association with MT1-MMP. The specificity of these procedures was demonstrated by the lack of signal in the absence of antibodies (Fig. 5*C, lanes 2* and *5*). A similar profile of MT1-MMP forms was previously reported by Lehti *et al.* (25) on the surface of phorbol ester-treated HT-1080 cells, which are known to express TIMP-2 (10, 19). These results also demonstrate that the processing and cellular localization of MT1-MMP forms in the vaccinia-infected cells is similar to that found in cells naturally expressing MT1-MMP.

We next examined the ability of the PM fractions derived from cells expressing MT1-MMP alone (containing the 60- and 44–40-kDa species) or MT1-MMP with TIMP-2 (containing the 60- and 57-kDa species) (shown in Fig. 5*A*) to initiate pro-MMP-2 activation as described under "Experimental Procedures." As shown in Fig. 5*D*, the PM fraction of cells expressing MT1-MMP alone was unable to initiate pro-MMP-2 activation over a 5-h period, as determined by the background activity measured with a fluorescence quenched substrate (38). In contrast, pro-MMP-2 incubation with PM derived from cells co-expressing MT1-MMP and TIMP-2 resulted in a 12-fold enhancement of MMP-2 activity over that measured with PM of cells expressing MT1-MMP alone. It should be mentioned that the PM fraction capable of activating pro-MMP-2 was derived from cells infected to express MT1-MMP with relatively low amounts of TIMP-2 (0.1 pfu/cell of vSC59-T2) since high levels of TIMP-2 expression resulted in PM fractions unable to generate MMP-2 activity (data not shown). Taken together, these results suggest that the 57-kDa species of MT1-MMP is required for pro-MMP-2 activation.

The 57-kDa Species of MT1-MMP Binds TIMP-2—Previous studies demonstrated that MT1-MMP could form a complex with TIMP-2 on the cell surface (10). Our studies indicated that three major forms of MT1-MMP (60-, 57-, and 44–40 kDa) were present on the surface of cells with the amount of the 57-kDa species increasing in the presence of TIMP-2. In addition, our studies with BS-C-1 cells co-infected to express MT1-MMP and TIMP-2 showed that these cells exhibited surface expression of TIMP-2 suggesting that the inhibitor was interacting with MT1-MMP. Therefore, we asked which of the MT1-MMP forms could bind TIMP-2. To this end, samples of various time points of a pulse-chase experiment were incubated with or without exogenous unlabeled TIMP-2 and then subjected to immunoprecipitation with anti-TIMP-2. As shown in Fig. 6*A*, the samples from the 0, 45-, and 90-min chase periods contained the 60-, 57-, and/or the 44–40-kDa forms of MT1-MMP, as demonstrated after immunoprecipitation with the pAb 437. Addition of unlabeled TIMP-2 and the anti-TIMP-2 mAb to each of these samples revealed that, under these conditions, only the ^{35}S-labeled 57-kDa form of MT1-MMP co-precipitated with the inhibitor (Fig. 6*A*). In the absence of TIMP-2, no signal was detected with the anti-TIMP-2 antibody (data not shown).

To further examine the nature of the MT1-MMP species capable of binding TIMP-2, we used an Affi-Gel 10-TIMP-2-affinity matrix. To this end, lysates were prepared from BS-C-1 cells expressing MT1-MMP alone since co-expression with TIMP-2 would have affected binding to the affinity matrix. As shown in Fig. 6*B*, the 57-kDa species bound to the TIMP-2-affinity matrix (Fig. 6*B, lane 1*) whereas the 60- and 44–40-

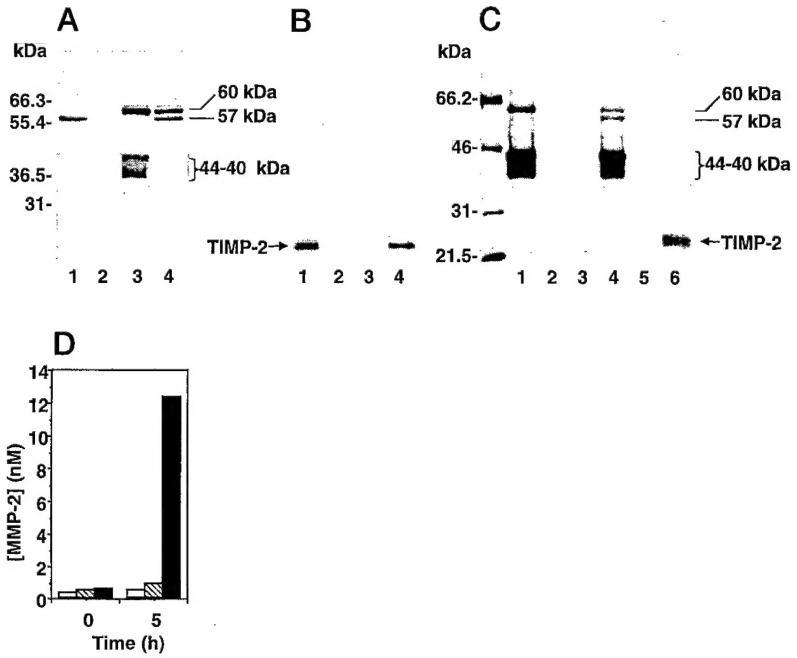

FIG. 5. **Effect of TIMP-2 on the PM localization and surface association of MT1-MMP forms.** *A* and *B*, immunoblot analysis of MT1-MMP and TIMP-2 in PM fractions. PM (12 μg/lane) isolated from BS-C-1 cells infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 to express MT1-MMP (*lane 3*) or co-infected with vT7-MT1 (5 pfu/cell) and vSC59-T2 (0.1 pfu/cell) to co-express MT1-MMP and TIMP-2 (*lane 4*) were subjected to reducing 10% SDS-PAGE followed by immunoblot analysis with pAb 437 to MT1-MMP (*A*) or with a mAb to TIMP-2 (*B*). As controls, PM isolated from BS-C-1 cells infected with the vTF7-3 virus alone (*lane 2*) and of phorbol ester-treated HT-1080 cells (16 μg) (*lane 1*) were analyzed in parallel. *C*, biotinylation of MT1-MMP forms in BS-C-1 cells co-infected to express MT1-MMP alone (*lanes 1–3*) or MT1-MMP and TIMP-2 (*lanes 4–6*) as described above. The lysates (500 μl) were immunoprecipitated with either the pAb 437 to MT1-MMP (*lanes 1* and *4*) or the mAb CA101 to TIMP-2 (*lanes 3* and *6*) and protein G-Sepharose beads. As controls, the lysates received protein G-Sepharose beads without antibody (*lanes 2* and *5*). The immunoprecipitates were resolved by reducing 12% SDS-PAGE followed by blotting to a nitrocellulose membrane and detection by streptavidin-horseradish peroxidase and ECL. The biotinylated molecular weight markers shown are from Bio-Rad. *D*, activation of pro-MMP-2 by PM. Pro-MMP-2 (55 nM), in collagenase buffer, was incubated at 37 °C in the absence (*open bar*) and presence of PM (0.15 μg/μl) isolated from BS-C-1 cells infected to express MT1-MMP (*hatched bar*) or co-express MT1-MMP and TIMP-2 (*black bar*), as described above (*A*). MMP-2 activity was measured at 0 and 5 h incubation as described under "Experimental Procedures." Similar results were obtained in two independent experiments.

kDa forms of MT1-MMP did not (Fig. 6*B*, *lane 3*) in agreement with the co-immunoprecipitation experiment. These results also show that although cells expressing MT1-MMP alone contain low levels of the 57-kDa species of MT1-MMP, this form can be enriched by the affinity step. The 57-kDa species failed to bind to the TIMP-2 affinity column in the presence of 5 mM EDTA (data not shown). No binding of MT1-MMP species was detected to the affinity matrix without TIMP-2 (Fig. 6*B*, *lane 2*). PM fractions isolated from phorbol ester-treated HT-1080 cells containing only the 57-kDa species were used as positive control (Fig. 6*B*, *lane 4*).

N Termini of the MT1-MMP Species—To define the nature of the MT1-MMP species including the TIMP-2-binding 57-kDa form, a lysate of BS-C-1 cells infected to co-express MT1-MMP and TIMP-2 to induce accumulation of the 57-kDa species was subjected to immunoaffinity purification as described under "Experimental Procedures." The 60-, 57-, and 44-kDa (upper band) MT1-MMP species were isolated from the lysates and subjected to N-terminal sequencing. As shown in Fig. 7, the isolated 57-kDa form of MT1-MMP displays a N terminus starting at Tyr^{112} and therefore is identical to the active species of MT1-MMP previously reported (10, 25). The N terminus of the 60-kDa species starts at Ser^{24} demonstrating that this species is the latent form (pro-MT1-MMP) (1). The 44-kDa form starts at Gly^{285}, which is at the beginning of the hinge region (1, 8), and therefore lacks the complete catalytic domain. In agreement with the N-terminal sequencing data, a pAb to the propeptide domain of pro-MT1-MMP (pAb 36) (34) failed to recognize both the 57- and 44-kDa species (data not shown).

DISCUSSION

A cellular approach designed to express MT1-MMP in the absence or presence of TIMP-2 facilitated the characterization of the three major MT1-MMP species and revealed a unique interaction between MT1-MMP and TIMP-2 by which the inhibitor regulates the nature of MT1-MMP enzymes on the cell surface. The results presented here demonstrate that the concentration of active MT1-MMP (57 kDa) on the cell surface was directly and positively regulated by TIMP-2. Absence of inhibitor, on the other hand, resulted in a significant decrease in the amount of active enzyme on the cell surface leading to the generation of a membrane-bound inactive 44-kDa species, which was further processed to lower molecular weight forms. N-terminal sequencing of the 44-kDa species revealed that this form starts at Gly^{285} and hence lacks the entire catalytic domain but maintains the hemopexin-like domain and the hinge region (1). A previous study reported a N-terminal sequence of a 43-kDa species of MT1-MMP starting at Ile^{256} in the catalytic domain (25). This species was isolated from the media of HT-1080 cells transfected to overexpress a soluble transmembrane-deleted MT1-MMP and therefore the enzyme and/or mechanism responsible for MT1-MMP cleavage in solution could not be established. Although both the previously reported 43-kDa form (25) and the 44-kDa species identified in the present study are functionally inactive, our sequencing data were obtained with wild type MT1-MMP and therefore the N terminus of the 44-kDa species is likely to represent a true cleavage site.

Previous studies implicated MMP-2 in the processing of active MT1-MMP to the 44-kDa species (40, 41). However, our results clearly show that this process is MMP-2 independent

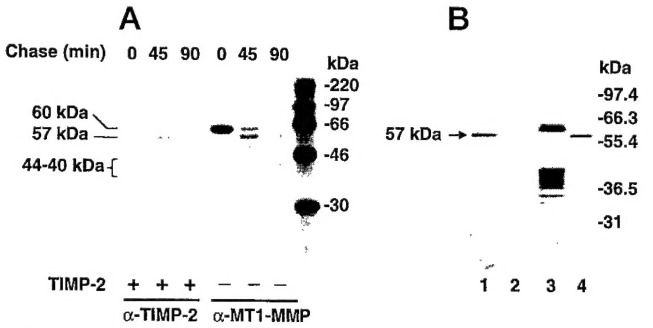

FIG. 6. **The 57-kDa form of MT1-MMP binds TIMP-2.** *A*, BS-C-1 cells were infected-transfected to express MT1-MMP as described in the legend to Fig. 1*A*. Three and half hours post-infection, the cells were pulse-labeled with [^{35}S]methionine for 15 min and chased for 90 min. At various times (0, 45, and 90 min), the cells were harvested in lysis buffer. A fraction of the lysates (20 μl) was incubated (2 h, 4 °C) with 150 nM unlabeled TIMP-2 (+) and then immunoprecipitated with the mAb to TIMP-2 (α-TIMP-2). In parallel, another fraction of the lysates (20 μl), which did not receive unlabeled TIMP-2 (−), was supplemented with harvest buffer to immunoprecipitate MT1-MMP with the pAb 437 (α-MT1-MMP), as described under "Experimental Procedures." The immunoprecipitates were resolved by 10% SDS-PAGE under reducing conditions followed by autoradiography. *B*, BS-C-1 cells were infected to express MT1-MMP. The next day, the cells were lysed in lysis buffer. The lysates (500 μl each) were incubated (12 h, 4 °C) with either Affi-Gel 10-TIMP-2 matrix (*lanes 1* and *3*) or Affi-Gel 10 matrix without immobilized TIMP-2 (*lane 2*) (50 μl suspension each). The bound fraction was eluted with 20 μl of Laemmli sample buffer with β-mercaptoethanol. The samples (bound and unbound) were resolved by 12% SDS-PAGE followed by immunoblot analysis with the pAb 437 to MT1-MMP. *Lane 1*, fraction bound to Affi-Gel 10-TIMP-2 matrix; *lane 2*, fraction bound to Affi-Gel 10 matrix; *lane 3*, unbound fraction from Affi-Gel 10-TIMP-2 matrix; and *lane 4*, PM of phorbol ester-treated HT-1080 cells, as control.

since vaccinia-infected BS-C-1 cells do not produce detectable pro-MMP-2 (26, 27). Cleavage at the Gly-Gly^{285} peptide bond and consequent generation of the 44-kDa species was significantly inhibited by TIMP-2, as shown in cells co-expressing MT1-MMP and TIMP-2. Ellebroeck *et al.* (40) reported a reduction of the 44-kDa species in concanavalin A-treated ovarian carcinoma cells receiving exogenous TIMP-2. However, the effects of TIMP-2 on the profile of the MT1-MMP forms could not be differentiated from the pleiotrophic effects of concanavalin A, which may include effects on TIMP-2 and/or MT1-MMP expression as well as effects on cellular organization and plasma membrane structure. A recent study indicated that TIMP-2 modulates MT1-MMP activity in melanoma cells. However, a direct correlation between TIMP-2 expression and MT1-MMP processing could not be established (42). Based on the results in the vaccinia system, which allowed modulation of the level of TIMP-2 expression in the cells, we can conclude that the accumulation of the 57-kDa species of MT1-MMP and the reduction in the amount of the 44-kDa forms is a sole consequence of the presence of TIMP-2. The fact that TIMP-2, but not TIMP-1, inhibited the generation of the 44-kDa species further demonstrates that MT1-MMP processing is an autocatalytic event. The lack of effect of TIMP-1 is consistent with the weak inhibitory activity of TIMP-1 for MT1-MMP, as previously reported (12, 43). However, the inhibitory effect of TIMP-2 can be mimicked by general synthetic MMP inhibitors (40, 41) or by exposure of cells to concanavalin A or phorbol ester, which may induce TIMP-2 expression.

Due to the difficulty in modulating the level of MT1-MMP and TIMP-2 expression in cells the direct contribution of TIMP-2 to the activation of pro-MMP-2 could not be established in a cell-based system. The data presented here show for the first time in a living cellular system that pro-MMP-2 activation by wild type MT1-MMP is tightly regulated by the level of

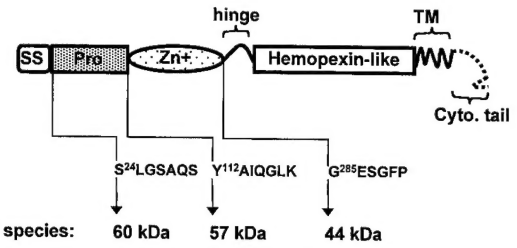

FIG. 7. **N-terminal sequence of MT1-MMP forms.** Diagram of the location and sequence of the MT1-MMP forms isolated. *SS*, signal sequence; *pro*, propeptide domain; *Zn*$^+$, catalytic domain; *TM*, transmembrane region; *Cyto. tail*, cytoplasmic tail.

TIMP-2 expression in the cells. These results are consistent with the biphasic role of TIMP-2 in pro-MMP-2 activation as first proposed by Strongin *et al.* (10) *in vitro*. The stimulatory effect of TIMP-2 on pro-MMP-2 activation has been attributed to the formation of a ternary complex between MT1-MMP, TIMP-2, and pro-MMP-2 (10, 18, 19). However, our data show that BS-C-1 cells expressing MT1-MMP alone were able to initiate pro-MMP-2 activation suggesting that TIMP-2, and hence ternary complex formation, may not be an absolute requirement for pro-MMP-2 activation. Consistently, a C-terminal truncated TIMP-2, unable to form a complex with pro-MMP-2, and marimastat, a synthetic MMP inhibitor, can promote, at relatively low concentrations, the accumulation of active MT1-MMP and consequently the activation of pro-MMP-2.[2] Under these conditions, the surface association of pro-MMP-2 is likely to be mediated by other factors, which may include stable structural elements and/or nonspecific adsorption. Previous studies have shown that the association of pro-MMP-2 with the cell surface can be mediated by a variety of factors including ECM components (44, 45), heparin (19), and/or the integrin $\alpha_v\beta_3$ (46).

Our findings suggest that the effects of TIMP-2 on pro-MMP-2 activation may also be related to its ability to control the amount of active MT1-MMP in the cells by preventing the autocatalytic processing of active MT1-MMP on the cell surface. This is based on the observation that co-expression of MT1-MMP with increasing levels of TIMP-2 produced a gradual accumulation of the 57-kDa species that correlated, at the lowest level of inhibitor, with enhanced pro-MMP-2 activation when compared with cells expressing MT1-MMP alone. Thus, by binding and inhibiting a fraction of active MT1-MMP, TIMP-2 reduces the extent of autocatalytic processing of free 57-kDa species and therefore promotes its accumulation on the cell surface, which in turn enhances pro-MMP-2 activation. However, the ratio between free and TIMP-2-bound (inhibited) active (57 kDa) MT1-MMP has yet to be determined. At high inhibitor concentrations, activation of pro-MMP-2 was significantly diminished despite the higher amounts of 57-kDa MT1-MMP detected under those conditions. A plausible explanation for this apparent inconsistency may be the titration of all active MT1-MMP species by TIMP-2, which results in inhibition of pro-MMP-2 activation as previously proposed (18, 19). Furthermore, the maximum pro-MMP-2 activation observed at the lowest level of the 57-kDa species detected in the cells may reflect the high catalytic efficiency of active and TIMP-2-free MT1-MMP toward pro-MMP-2. This may also be the case in cells infected to express only MT1-MMP. Our data suggest that the 57-kDa species of MT1-MMP is the major pro-MMP-2 activator. This species is the active enzyme form, as determined by N-terminal sequencing, and its appearance correlated with

[2] M. Toth, D. Gervasi, Y. A. De Clerck, and R. Fridman, unpublished results.

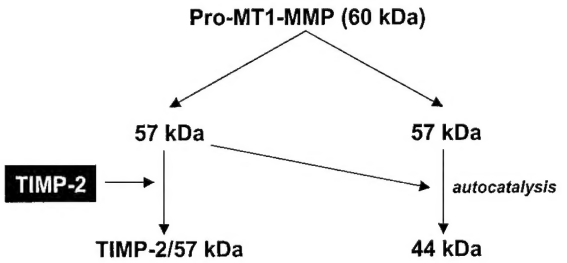

FIG. 8. **Diagram of MT1-MMP processing and interactions with TIMP-2.**

enhanced pro-MMP-2 activation. In addition, we have shown that only PM fractions containing the 57-kDa species (derived from cells co-expressing MT1-MMP and TIMP-2) were able to initiate pro-MMP-2 activation whereas PM fractions lacking the 57-kDa species had no activity toward pro-MMP-2. Finally, the 57-kDa species co-migrated with the 57-kDa species of MT1-MMP detected in PM fractions of phorbol ester-treated HT-1080 cells, which were shown to activate pro-MMP-2 (10, 37, 47) and to contain mostly active MT1-MMP (10, 19).

The nature of the MT1-MMP species responsible for TIMP-2 binding remains controversial. Both latent (21, 22) and active species (10) of MT1-MMP have been implicated in binding to the inhibitor. The data presented here are consistent with the 57-kDa species of MT1-MMP being the major TIMP-2 binding form. Co-immunoprecipitation and TIMP-2 affinity experiments demonstrated that the 57-kDa species of MT1-MMP bound to TIMP-2, whereas pro-MT1-MMP and the 44-kDa form showed no binding under the same conditions suggesting that the latter forms exhibit a reduced affinity for TIMP-2. Considering that the 57-kDa species starts at Tyr^{112} and that pro-MT1-MMP showed no significant TIMP-2 binding, it is reasonable to assume that TIMP-2 interacts with the active site of MT1-MMP (48). Furthermore, the binding of TIMP-2 to the 57-kDa species was abolished in the presence of EDTA, suggesting that the catalytic Zn^{2+} ion is required for TIMP-2 binding. These results are not consistent with the propeptide domain of pro-MT1-MMP being required for TIMP-2 binding (22).

Fig. 8 depicts a model of MT1-MMP processing and its regulation by TIMP-2. Cells produce pro-MT1-MMP that is activated by a pro-convertase-dependent mechanism (14, 49). Activation may occur in the trans-Golgi network and/or at the cell surface, as furin is also targeted to the PM and to the extracellular space (50, 51). Surface-anchored active MT1-MMP (57 kDa) is a fully functional enzyme capable of pericellular proteolysis. However, in the absence of TIMP-2, the 57-kDa species undergoes autocatalytic conversion to a 44-kDa inactive membrane-tethered form by cleavage at the hinge region (Gly^{285}), which diminishes the surface availability of active MT1-MMP. The 44-kDa form is further processed to a series of lower molecular weight degradation products. Under these conditions, the extent of MT1-MMP-dependent proteolysis would be determined by a balance between pro-MT1-MMP expression and activation, the rate of autocatalytic processing and enzyme internalization. Binding of TIMP-2 to activated MT1-MMP (57 kDa) inhibits autocatalysis and consequently, active enzyme accumulates on the cell surface as cells produce more MT1-MMP. This suggests that, under conditions of low levels of TIMP-2 expression relative to MT1-MMP, TIMP-2 may act as a positive regulator of MT1-MMP activity and therefore may enhance pericellular proteolysis including pro-MMP-2 activation and ECM degradation. The 57-kDa·TIMP-2 complex also favors the localization of pro-MMP-2 (by ternary complex formation) on the cell surface thereby promoting pro-MMP-2 activation (10, 19). However, other pro-MMP-2-cell surface interaction could play a role (19, 44, 46). Excess TIMP-2, relative to MT1-MMP, will eventually block all active MT1-MMP inhibiting proteolysis. This model reflects the dynamic and unique interactions between MT1-MMP and TIMP-2 in living cells that tightly regulate MT1-MMP pericellular activity.

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